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16. ABSTRACT

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Effects of Hemorrhagic Stress On Several Blood Parameters in Adult Rainbow Trout (*Salmo gairdneri*)

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ABSTRACT

Blood was removed from ten adult rainbow trout (*Salmo gairdneri*) on a sequential daily (7 days), weekly (4 weeks), and monthly (1 month) schedule and analyzed for hematocrit, plasma protein, acid phosphatase (AP), lactic dehydrogenase (LDH), and creatine phosphokinase (CPK). Of the parameters examined, hematocrit, lactic dehydrogenase, and creatine phosphokinase were found to be the most significant indicators of stress due to repeated sampling. We showed that, under conditions similar to those of this investigation, daily and weekly sampling of approximately 0.2% of the body weight in blood produced decidedly deleterious effects of the test organisms. Starvation was a secondary stressor and is believed to have had an influence on blood characteristics as the study entered its later phases.

The use of routine hematological methods as aids in diagnosis has benefited higher vertebrate medicine. Fish health research and cultural operations are finding application of these same techniques beneficial. By using blood chemistry values as indicators of sublethal stress, clinical tests with fish may prove to be valuable diagnostic tools. Some efforts in this direction have been made by Blaxhall (1972), and Bouck and Ball (1966) found significant differences in blood characteristics of rainbow trout captured by hook-and-line, seine, and electroshock. These findings were partially disputed by Schreck et al. (1976) in a study of the physiological effects of electroshock on rainbow trout. Stress of anesthetization with unbuffered narcotic agents has also been shown to induce significant alteration in the blood chemistry of salmonids (Houston et al. 1971a, 1971b; Wedemeyer 1970). Barnhart (1969) examined diet, strain, age, water temperature, sampling technique, and test methods as variables affecting hematological characteristics of rainbow trout and found important differences with all variables. Houston and DeWilde (1968) concluded that hematocrit values may be substituted for erythrocyte counts and hemoglobin determinations in routine evaluations of hematological status.

In extended pollution and hematological studies, blood analysis usually requires repeated sampling. Various cannulation methods have been used to allow repeated blood collection from unanesthetized fish (Smith and Bell 1964; Garey 1969; Houston 1971; Soivio and Nyholm 1975). Although Klontz and Smith (1968) have indicated that blood volumes equivalent to 1% of the body weight can be withdrawn weekly from salmonid fishes, little is known about the hematological effects caused by frequent and repeated blood sampling or the effects on recovery time.

The objective of the present study was to determine the effects of hemorrhagic stress, caused by frequent and repeated blood sampling, on the following blood parameters in adult rainbow trout (*Salmo gairdneri*): (1) hematocrit; (2) total plasma protein; (3) plasma acid phosphatase (AP) activity; (4) plasma lactic dehydrogenase (LDH) activity; and (5) plasma creatine phosphokinase (CPK) activity. The activity of these three enzymes has been used at Western Fish Toxicology Station as a battery of tests to determine effects, if any, of various sublethal stressor agents on salmonid fish. The effects of blood sampling on these parameters were assessed until some mortality occurred and through recovery of the survivors. The study was viewed with the thought of using these characteristics as indicators of environmental stress in salmonid health research. Data reported in this paper may

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be useful in establishing appropriate sampling intervals in subsequent investigations.

METHODS

Ten adult rainbow trout were randomly assigned to individual circular fiberglass tanks on 13 July 1976. The fish were uniform in size with a mean weight of 1,500 g and mean length of 43 cm. The test fish were fed Oregon Moist Pellets to repletion but were not fed from 1 day prior to the test until day 43 of the experiment. This feeding schedule was chosen to eliminate the effects of food on blood parameters during the daily and weekly sampling phases. Daily feeding was resumed from day 43 until the test was terminated on day 63, although no food was offered during the 24 hours prior to blood sampling.

The tanks, equipped with flow-through water systems, were 1.3 m in diameter, 76.2 cm deep, contained about 500 liters of aerated water, and had a flow rate of 11 liter/min. Translucent tank covers allowed a natural photoperiod. Sleeved center standpipes maintained the water level and aided in cleaning wastes from the bottom of the tanks. Temperature of the test water, taken from wells near the Willamette River, fluctuated between 13.5 and 17.5 C during the test period. Total alkalinity ranged from 18 to 24 ppm as $CaCO_3$, total hardness from 21 to 24 ppm as $CaCO_3$, and pH from 6.7 to 7.2. More complete well-water chemistry data are contained in a publication by Samuelson (1976).

Blood Collection

On the first day all fish were electroshocked with 200 volts DC current prior to narcotization with neutralized tricaine methanesulfonate (M.S. 222) to stage III, plane 2 as described by Klontz (1964). On all subsequent sampling dates, the electroshock treatment was dispensed with; the fish were gently netted and placed directly into the neutralized anesthetic solution. Although the use of electroshock on day 1 is inconsistent with the capture method used on subsequent days, Schreck et al. (1976) demonstrated that shocking does not significantly affect hematocrit, plasma protein, or enzyme patterns. Three-ml sterile, nonwettable polyethylene syringes with 20-gauge × 38-mm hypodermic needles were used for blood collection. Plastic syringes were used to prevent decreased coagulation time (Smith et al. 1952). Each syringe was partially filled with a 10% solution of trisodium citrate as an anticoagulant, then all citrate was expelled except for the amount remaining in the needle which was calculated to be about 0.036 ml. Dilution factors were derived and blood volumes were corrected accordingly. In most instances, 3 ml of blood were withdrawn via dorsal aortic puncture by the method of Schiffman (1959). Any bleeding from the puncture wounds ceased within a minute. At no time did this bleeding significantly affect volume of blood withdrawn. Microhematocrit tubes were immediately filled from the syringes and the remainder of the blood was expelled into glass culture tubes which were then covered with plastic caps. Blood samples were collected daily for 1 week, weekly for the next 4 weeks, and once again 4 weeks later. Since two of the ten specimens died after sampling daily for 7 days (two others died on day 35), it appeared that the test fish were, in general, approaching death. We were interested in observing blood parameters during recovery as well as during the stress period so we chose this particular sampling schedule.

Blood Analysis

The microhematocrit tubes were centrifuged for 15 minutes and packed cell volumes determined with a microcapillary reader. The whole blood in the culture tubes was centrifuged for 10 minutes and the plasma pipetted off into glass culture tubes for analyses. The plasma was refrigerated while tests were prepared. All analyses were carried out within 20 hours of sampling.

Total plasma protein concentrations were determined by using a modification of Kingsley's (1942) Biuret method as follows: 50 μl of plasma were mixed with 2 ml of Biuret reagent. After 30 minutes reaction time, the absorbance was measured[2] and

[2] Plasma protein and enzyme activities were determined colorimetrically, using a Bausch and Lomb Spectronic 88 spectrophotometer (Bausch and Lomb Co.).

TABLE 1.—*Summary of the effects of hemorrhagic stress on rainbow trout blood parameters, presenting means and standard errors of the means (in parentheses) for each sampling day. PCV = packed cell volume; AP = acid phosphatase; LDH = lactic dehydrogenase; CPK = creatine phosphokinase.*

Day	Hematocrit (% PCV)	Protein (g/100 ml)	Activity units per 50 µl plasma			Activity units per g protein		
			AP[a]	LDH	CPK	AP[b]	LDH[c]	CPK[c]
1	37.3	4.4	4.5	0.63	0.04	2.0	2.90	0.19
	(1.7)	(0.1)	(1.0)	(0.03)	(<0.01)	(0.4)	(0.16)	(0.02)
2	31.6	3.7	3.1	0.74	1.7	1.6	3.96	9.02
	(1.0)	(0.1)	(0.2)	(<0.01)	(0.3)	(0.1)	(0.08)	(1.50)
3	26.7	3.5	4.5	2.35	3.6	2.6	13.70	20.87
	(0.8)	(0.1)	(0.4)	(0.43)	(1.2)	(0.2)	(2.89)	(6.69)
4	22.0	3.6	3.5	2.51	3.2	1.9	14.19	17.92
	(2.5)	(0.1)	(0.4)	(0.62)	(0.9)	(0.2)	(3.81)	(5.54)
5	18.4	3.7	2.8	2.16	2.6	1.5	11.76	14.14
	(1.1)	(0.1)	(0.2)	(0.45)	(0.7)	(0.1)	(2.50)	(3.86)
6	14.9	3.5	2.7	2.26	3.3	1.6	13.34	19.25
	(1.8)	(0.1)	(0.5)	(0.36)	(0.8)	(0.3)	(2.59)	(5.20)
7	12.6	3.6	3.7	1.86	4.6	2.1	9.75	25.85
	(1.2)	(0.1)	(0.5)	(0.40)	(1.0)	(0.3)	(2.38)	(6.46)
14	20.4	4.0	2.4	0.78	1.5	1.2	4.00	8.05
	(0.6)	(0.1)	(0.4)	(0.08)	(0.3)	(0.2)	(0.52)	(1.70)
21	17.8	3.6	2.6	0.55	1.9	1.4	3.03	9.98
	(1.0)	(0.1)	(0.4)	(0.06)	(0.6)	(0.2)	(0.32)	(2.61)
28	15.5	3.8	2.7	0.64	2.5	1.4	3.41	13.44
	(1.5)	(0.1)	(0.3)	(0.12)	(0.8)	(0.1)	(0.59)	(4.32)
35	13.4	3.8	2.8	0.80	4.1	1.5	4.22	21.13
	(1.2)	(0.1)	(0.3)	(0.16)	(1.5)	(0.2)	(0.76)	(8.75)
63	10.5	3.5	2.7	1.10	0.43	1.6	6.42	2.65
	(3.2)	(0.2)	(0.3)	(0.30)	(0.14)	(0.2)	(1.93)	(0.88)

[a] ($\times 10^{-5}$); [b] ($\times 10^{-8}$); [c] ($\times 10^{9}$).

converted to equivalent grams of bovine serum albumin/100 ml.

Enzyme analyses were conducted with reagents prepared by Sigma Chemical Company and methods described in the Sigma Technical Bulletins (Sigma Chemical Company 1975a, 1975b, 1975c). Enzyme activities were expressed as follows: acid phosphatase, as µM of p-nitrophenol produced/50 µl of plasma; lactic dehydrogenase, as µM of pyruvate used/50 µl of plasma; and creatine phosphokinase, as µM of creatine produced/50 µl of plasma. All enzyme activities were also expressed as activity units/gram of plasma protein. Means and standard errors of the means were calculated on each sampling date and individual values for each fish were recorded through the test period.

Physical Measurements

After conclusion of the test, the six surviving test fish were weighed and measured. As another indication of stress on the test fish, they were compared with non-test fish from the same original population which were also weighed and measured at the same time. Non-test fish were fed throughout the period.

RESULTS

As Table 1 demonstrates, percent packed cell volumes were highly affected by hemorrhagic stress. During the daily sampling phase, the mean values decreased by 66%, from 37.3 to 12.6. During the weekly sampling phase, after an initial increase to 20.4, the mean values decreased steadily to 13.4. After the 4-week phase, the hematocrit had further declined to 10.5. Figure 1 shows that fish #8 was the lone exception to this trend. An overall decrease of 72% in hematocrit values occurred during the 63-day test period. As a measure of variability, the ratio of mean/standard error of the mean, using both activity/50 µl and activity/g protein, averaged 9.

Plasma protein demonstrated very little variation throughout the test period. After a decrease from 4.4 to 3.7 g/100 ml between

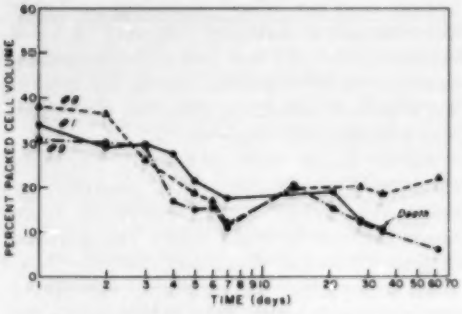

FIGURE 1.—*Effects of hemorrhagic stress on hematocrit values of individual rainbow trout.*

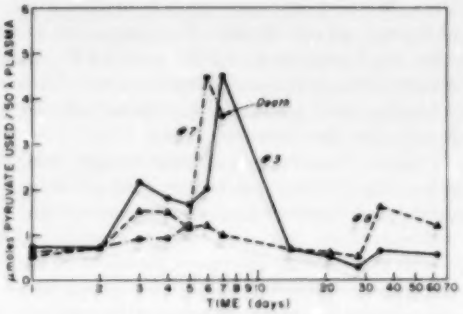

FIGURE 2.—*Effects of hemorrhagic stress on plasma lactic dehydrogenase activity of individual rainbow trout.*

days 1 and 2, total plasma protein did not vary by more than 5% during the daily sampling phase of the test (Table 1). During the weekly sampling phase plasma protein increased from 3.6 to 4.0 g/100 ml, then decreased slightly. After the 1-month recovery period, the protein again had decreased, from 3.8 to 3.5 g/100 ml. The variability coefficient averaged 3 during the test.

Acid phosphatase activity was quite variable throughout the study period (Table 1) although the overall result was a 40% decrease in activity between days 1 and 63. The variability coefficient averaged 13. AP activity reached its lowest level on day 14 at 57% of the initial activity.

Table 1 shows that LDH activity was highly correlated with the stress placed on the fish. Activity increased fourfold during the daily sampling phase, reaching a peak of 2.51 units on day 4. After a decrease of 70% between days 7 and 21, activity then increased with a gain of 100%. Although the final activity was 75% higher than the initial activity, it was considerably lower than the maximum activity. The variability coefficient averaged 17. Figure 2 shows LDH activity of three of the test fish.

The CPK activity was the parameter most highly affected by hemorrhagic stress. The activity of the enzyme had increased 114-fold after the 1 week of daily blood sampling (Table 1). During the weekly sampling phase, an initial 67% decrease in CPK activity was observed between days 7 and 14, followed by a steady increase of 173% between days 14 and 35. Although the final activity/50 μl was more than ten times the initial activity, it was only 9% of the peak activity. As with LDH activity, unexpected decreases in CPK activity occurred within the daily sampling phase. The variability coefficient averaged 27. This parameter showed the greatest variability among individual fish on any given sampling day.

All ten specimens developed an extensive darkening of one side of the head which we speculate was caused by cerebral ischemia. The fish became sluggish and easy to net as the project advanced. Net avoidance increased during recovery.

Although Oregon Moist Pellets were offered to the six remaining fish during the recovery phase of the test, only fish #8 was observed accepting food. This fish consumed pellets voraciously on a daily basis until the test was terminated. The other five fish failed to eat when pellet food was offered.

Weight, length, and condition factors of surviving test fish were compared with those of non-test fish from the same original population. In the tested group, although length was depressed only 8.3%, condition factor was reduced by 13.4% and total weight was depressed by 33.1%.

DISCUSSION

Results of this study demonstrate that hemorrhagic stress, induced by frequent and repeated blood sampling, has a pronounced effect on certain blood character-

istics. We feel that starvation constituted an additional stress factor. Discussion of results on hematocrit, LDH, and CPK will demonstrate this secondary stressor. Lack of feeding and low condition factor also indicate that starvation occurred.

Precise diagnosis of anemia results from detection of reduction below normal in the number of erythrocytes and/or hemoglobin concentration per unit volume of blood (Smith et al. 1972). However, because of the correlation between these two properties and percent packed cell volume, hematocrit is a useful method for the detection of ordinary anemia in fish (Wells 1956; Snieszko 1960). Our results are consistent with this determination. The repeated sampling apparently produced anemia in the test organisms, demonstrated by the drastic decline in hematocrit values and the moribund appearance of the fish. All of the individuals exhibited an increase in packed cell volumes during the first week or two of the weekly sampling phase and all but one fish showed subsequent decreases in hematocrit after this initial recovery. The one fish that accepted food showed an increase of 19% in hematocrit value during the 1-month recovery phase, due, probably, to recovery from starvation.

No hematocrit value can be pinpointed as the lethal level for adult rainbow trout. Fish #9 reached the same depressed packed cell volume as fish #1 on day 35 (and even declined further during the following month: Fig. 1). The latter fish died whereas the former survived until the test was terminated.

LDH and CPK are both useful in the diagnosis of myocardial damage in humans (McDonald et al. 1957; Cook 1967) and CPK is also used as an indicator of cerebral infarction in humans (Eisen and Sherwin 1968). The physical appearance of these trout suggests that this condition may have been present. Because rainbow trout brain tissue is high in CPK activity (Cairns and Christian, unpublished data), it is suggested that the elevated plasma CPK levels were caused by cellular leakage.

Mean CPK activity exhibited an initial decrease (recovery) between days 7 and 14, but a steady increase (stress) in activity between days 14 and 35 (Table 1). A 1-week period between blood sampling probably does not allow sufficient recovery in adult rainbow trout. All test fish demonstrated a decrease in CPK activity during the final 4-week period indicating that this time span was adequate for recovery.

AP activity was extremely variable throughout the study period, possibly because AP is extremely unstable at room temperatures or even when refrigerated (Sigma Chemical Company 1975a). However, we feel that the variability was due to the very low levels of activity present in the plasma. The greatest difference observed (between days 1 and 14) was 0.000021 μM. Optical densities for AP analysis were consistently less than 0.10 and frequently as low as 0.001. This raises a question as to the effectiveness of AP analysis as it is used here.

The unexpected declines (recovery) in LDH and CPK activity on days 4, 5, and 7 are difficult to explain. A possible explanation lies in the general phenomenon of homeostatic mechanisms in fish.

Larsson et al. (1976) found that among marine teleosts, hematocrit levels are positively correlated with the activity of the species and that total plasma protein concentrations showed some relationship to activity. It is not known whether short-term limitations of activity would induce changes in these blood parameters. If so, the size of tanks used in this study may have represented an additional consideration by lowering the level of activity of the test fish.

In hindsight, it would have been informative to have used more fish and anesthetized and handled some of them without withdrawing blood until the end of the test, thereby assessing effects of these procedures by themselves. However, considering the methods used, we feel that the trends in blood parameter changes are useful information for fishery researchers. The lack of changes in plasma protein and enzyme levels resulting from electroshock is documented by Schreck et al. (1976). Anesthetization with buffered M.S. 222 does not produce changes in several blood parameters of rainbow trout, as Wedemeyer (1970) has shown. Bouck and Ball (1966) demonstrated that plasma protein concentration is

reduced by stressful methods of capture and handling. All of the protein values during our study were within the normal range for rainbow trout as described by Wedemeyer and Chatterton (1970) and no significant hypoproteinemia was observed. We therefore suggest that our capture, anesthetization, and handling procedures did not significantly alter blood parameters studied, and that withdrawal of blood represented the principal stressor.

When long-term projects utilizing hematological techniques are pursued, effects of sampling must be considered. This study demonstrates that hemorrhagic stress is a necessary consideration in experimental designs of studies using repeated blood sampling from the same rainbow trout. It is likely that other species would be similarly affected.

Although the total blood volume withdrawn from each fish was substantial over the course of the test, this volume was always replaced between sampling days. Following removal of a considerable amount of blood, full replenishment of the erythrocytes requires 4 to 6 weeks; leukocytes are replaced in 1 or 2 weeks; fluid volume is replaced within an hour or two (Smith et al. 1972). Therefore, although hypovolemia is not aggravated even by daily sampling, full replacement of vital blood constituents will not occur if a considerable amount of blood is withdrawn at intervals of less than 1 month.

At least two questions are raised by these considerations. (1) Could hemorrhagic stress by minimized by withdrawing smaller blood volumes or is the stress a function of repeated sampling and not the volume sampled? (2) If daily sampling is too frequent, what interval would allow adequate recovery to prevent hemorrhagic stress? Our sampling procedure involved removing blood volumes equivalent to 0.2% (3 ml of blood = about 3 g/1,500 g fish) of the body weight at daily, weekly, and monthly intervals. Based on data of Smith (1966), salmonid blood volume equals approximately 6% of the body weight. Therefore, the quantity sampled in this test was about 3.3% of the total blood volume on each sampling day. The data dispute the contention of Klontz and Smith (1968) that blood volumes equivalent to 1% of the body weight can be withdrawn weekly from salmonids. Two fish died after weekly sampling in addition to the two which died after daily sampling. Until these questions are answered, studies involving frequent, repeated blood sampling from the same fish should be viewed with caution.

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